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Magnesium and the Role of *mgtC* in Growth of *Salmonella typhimurium*

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Salmonella typhimurium **has three distinct transport systems for Mg^{2+}: CorA, MgtA, and MgtB. The *mgtCB* operon encodes two proteins, MgtC, a hydrophobic protein with a predicted molecular mass of 22.5 kDa, and MgtB, a 102-kDa P-type ATPase Mg^{2+} transport protein. The *mgtCB* locus has been identified as part of a new *Salmonella* pathogenicity island, SPI-3. Transcription of *mgtCB* is regulated by extracellular Mg^{2+} via the two-component PhoPQ regulatory system important for virulence. To elucidate MgtC's role in a low-Mg^{2+} environment, we looked at growth and transport in strains lacking the CorA and MgtA Mg^{2+} transporters but expressing MgtB, MgtC, or both. *mgtC mgtB*$^+$ and *mgtC*$^+$ *mgtB*$^+$ strains exhibited growth in N minimal medium without added Mg^{2+} with a 1- to 2-h lag phase. An *mgtC*$^+$ *mgtB* strain was also able to grow in N minimal medium without added Mg^{2+} but only after a 24-h lag phase. In N minimal medium containing 10 mM Mg^{2+}, all strains grew after a short lag phase; the *mgtC*$^+$ *mgtB* strain grew to a higher optical density at 600 nm than an *mgtC*$^+$ *mgtB*$^+$ strain and was comparable to wild type. The lengthy lag phase before growth in an *mgtC*$^+$ *mgtB* strain was not due to lack of expression of MgtC. Western blot analysis indicated that substantial MgtC protein is present by 2 h after suspension in N minimal medium. Surprisingly, in an *mgtC*$^+$ *mgtB*$^+$ strain, MgtC was undetectable during Mg^{2+} starvation, although large amounts of MgtB were observed. The lack of expression of MgtC is not dependent on functional MgtB, since a strain carrying a nonfunctional MgtB with a mutation (D379A) also did not make MgtC. Since, during invasion of eukaryotic cells, *S. typhimurium* appears to be exposed to a low-pH as well as a low-Mg^{2+} environment, the growth of an *mgtC*$^+$ *mgtB* strain was tested at low pH with and without added Mg^{2+}. While significant quantities of MgtC could be detected after suspension at pH 5.2, the *mgtC*$^+$ *mgtB* strain was unable to grow at pH 5.2 whether or not Mg^{2+} was present. Finally, using $^{63}Ni^{2+}$ and $^{57}Co^{2+}$ as alternative substrates for the unavailable $^{28}Mg^{2+}$, cation uptake could not be detected in an *mgtC*$^+$ *mgtB* strain after Mg^{2+} starvation. We conclude that MgtC is not a Mg^{2+} transporter and that it does not have a primary role in the survival of *S. typhimurium* at low pH.**

In *Salmonella typhimurium*, there are three transport systems for Mg^{2+}, CorA, MgtA, and MgtB. The *corA* gene is constitutively expressed, and the encoded 40-kDa protein product mediates both the influx and efflux of Mg^{2+} (10, 11, 23). The *mgtA* and *mgtCB* loci encode putative P-type ATPases (10, 13, 22). Although found in prokaryotes, MgtA and MgtB share greater similarity to the eukaryotic P-type ATPases, in particular to the sarcoplasmic reticulum Ca^{2+}-ATPases, than to other prokaryotic P-type ATPases. Both loci are under control of the two-component PhoPQ regulatory system. PhoQ is a membrane sensor-kinase that at low Mg^{2+} concentrations phosphorylates PhoP to activate a variety of genes including *mgtA* and the *mgtCB* operon (8, 14, 15). Recent evidence has suggested, however, that there may be an additional mechanism for transcription of the *mgtA* locus (28). MgtA and MgtB, unlike CorA, mediate only the influx of magnesium and are produced under Mg^{2+}-limiting conditions.

The *mgtCB* locus encodes two proteins. MgtC is a hydrophobic protein with a predicted molecular mass of 22.5 kDa (25, 28). MgtB, the P-type ATPase, has a molecular mass of 102 kDa (22, 25). Although MgtB exhibits extensive sequence homology to members of the P-type ATPase superfamily, MgtC does not exhibit significant sequence homology to any proteins of known function in the current databases. Recently, the *mgtCB* locus has been identified as part of a new pathogenicity island, SPI-3 (3). Pathogenicity islands are segments of DNA containing virulence genes that are found in pathogenic organisms but absent from phylogenetically related nonpathogenic organisms. The *mgtC* gene is not necessary for *S. typhimurium* invasion or short-term survival in epithelial or macrophage cells (24, 28). It is, however, essential for long-term survival within the macrophage and for virulence in mice (3). A functional allele of *mgtA* is not required for survival within the macrophage, while inactivation of *mgtB* has only a small effect on survival. Since addition of 25 mM Mg^{2+} to the macrophage growth medium rescued the ability of an *mgtC* mutant strain to survive long-term within the macrophage, it was suggested that MgtC may be an additional Mg^{2+} transporter in *S. typhimurium* (3).

In addition to a low Mg^{2+} concentration, *S. typhimurium* bacteria that invade epithelial and macrophage cells are also exposed to acid pH (1, 6, 7, 18). Exposure to acid also regulates gene expression of *mgtA* and *mgtCB* (2, 28). At pH 7.4, suspension of cells in N minimal medium without added Mg^{2+} induces transcription of *mgtA* and *mgtCB* more than 1,000-fold. At pH 5.2, low concentrations of Mg^{2+} fail to induce *mgtA*, but *mgtCB* induction is decreased only about twofold. If cells are adapted to pH 5.2 prior to Mg^{2+} starvation, there is no difference in *mgtA* or *mgtCB* induction properties compared to exposure at pH 7.4. Interestingly, a functional *corA* allele had a significant effect on transcription of these two loci. In strains lacking CorA and grown overnight at pH 5.2, induction of transcription at low Mg^{2+} levels was significantly diminished for both the *mgtA* and *mgtCB* loci. These data suggest that there is an acid component to induction of *mgtA* and *mgtCB* at

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TABLE 1. Bacterial plasmids and strains used in this study

Plasmid or strain	Relevant genotype	Source
Plasmids		
pDGK1	*mgtC*$^+$ *mgtB*$^+$	This study
pDGK12	*mgtC*$^+$ *mgtB* (D379A)	S. Roof and D. Kehres[a]
pMBM19	*mgtC*$^+$	This study
pMBM34	*mgtB*$^+$	This study
Strains		
MM196	Δ*leuBCD485 mgtC33*::Mu dJ	Hmiel et al. (10)
MM197	Δ*leuBCD485 mgtB11*::Mu dJ	Hmiel et al. (10)
MM281	Δ*leuBCD485 mgtB10*::Mu dJ *corA45*::Mu dJ *mgtA21*::Mu dJ *zjh1628*::Tn(Cam^r)	Hmiel et al. (10)
MM1265	*mgtC9232*::Mu dJ Δ*corA*	R. L. Smith[b]
MM1266	*mgtC9232*::Mu dJ	E. A. Groisman[c]
MM1269	*S. typhimurium* 14028s (wild type)	ATCC[d]
MM1442	LT2	ATCC
MM1490	MM281/pDGK1	D. Kehres[a]
MM1542	MM281/pDGK12	D. Kehres[a]
MM1648	MM1269/*mgtB11*::Mu dJ	This study
MM1665	MM281/pMBM19	This study
MM1733	MM281/pMBM34	This study
MM1737	MM1266/pMBM19	This study

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[c] Department of Molecular Microbiology, Washington University School of Medicine, St. Louis, Mo.
[d] ATTC, American Type Culture Collection.

low Mg^{2+} and that a functional *corA* allele is required for an optimal response to low Mg^{2+}. In this context, it is of interest that Bearson et al. (2) have recently presented evidence that PhoPQ regulation of these loci is also responsive to acid.

To elucidate MgtC's role in a low-Mg^{2+} environment, we compared growth and Mg^{2+} transport of a strain dependent on the *mgtCB* operon for Mg^{2+} uptake with a strain with an intact *mgtC* gene but with *mgtB* inactivated. In Mg^{2+}-starved cells, although Western blot analysis indicated that MgtC is produced and maintained, an *mgtC*$^+$ strain exhibited no detectable uptake of $^{63}Ni^{2+}$ or $^{57}Co^{2+}$. This strain could grow at pH 7.4 in the absence of added Mg^{2+} only after a 24-h lag period and could not grow regardless of the level of extracellular Mg^{2+} at pH 5.2. We interpret these results to indicate that MgtC is not a functional Mg^{2+} transporter and that its expression does not facilitate survival at low pH.

MATERIALS AND METHODS

Bacterial strains and plasmid construction. The molecular biology methods used were those of Sambrook et al. (21). Bacterial plasmids and strains used in this study are listed in Table 1. To create a *mgtC*$^+$-only plasmid, pDGK1 (11a) containing *mgtCB* in a pBS II SK+ (Stratagene, La Jolla, Calif.) vector was digested with *Hin*dIII. The 5.1-kb fragment was religated together to give pMBM19. To create a *mgtB*$^+$-only plasmid, pDGK1 was digested with *Nsi*I and *Bsu*36I, the ends were filled in with Klenow fragment, and the 7.3-kb fragment was religated to give pMBM34.

Growth curves. N minimal medium (10) was routinely supplemented with 0.4% (vol/vol) glucose as a carbon source and 0.1% (vol/vol) Casamino Acids. Strains were grown in 5 ml of N minimal medium supplemented with 10 mM Mg^{2+} and the appropriate antibiotics overnight at 37°C. Cell pellets were washed three times in N minimal medium without added Mg^{2+} and then resuspended in 5 ml of N minimal medium without added Mg^{2+}. The volume was adjusted slightly to give the same optical density at 600 nm (OD_{600}) for each strain. To 100 ml of N minimal medium, 500 μl of the cell sample was added, and then 7 ml of this mixture was added to each one of 10 test tubes. Ten milliliters was reserved and 200 μl of 1 M Mg^{2+} was added to give a 20 mM Mg^{2+} solution. Seven milliliters of this solution was added to the first test tube (already containing 7 ml of the cell culture), and the sample was mixed thoroughly to give a [Mg^{2+}] of 10 mM. Serial dilutions of Mg^{2+} were made by removing 7 ml of culture from the first test tube and adding it to the second test tube, etc., until the final test tube contained 10 μM Mg^{2+}. The tubes were incubated at 37°C, and the OD_{600} was measured at various time points. For comparison of growth at neutral and acid pH, the 100 mM Tris-Cl buffer in N minimal medium was replaced with 50 mM Na-HEPES plus 50 mM Na-MES (morpholineethanesulfonic acid), and the pH was adjusted appropriately.

Transport of $^{57}Co^{2+}$ and $^{63}Ni^{2+}$. Strains were grown in 5 ml of N minimal medium supplemented with 10 mM Mg^{2+} and the appropriate antibiotics for 4 h at 37°C. This culture was used to inoculate 200 ml of N minimal medium containing 10 mM Mg^{2+} and grown overnight at 37°C. The cells were centrifuged at 1,000 × *g* for 10 min and washed three times with the same volume of N minimal medium without added Mg^{2+}. Cells were resuspended in 500 ml of N minimal medium without added Mg^{2+} and grown at 37°C. At various time points, 25 ml of culture was centrifuged at 1,000 × *g* and washed once with N minimal medium without added Mg^{2+}. Cells were resuspended in N minimal medium to give an OD_{600} of 1.0 for use in the transport assay. The transport assay was performed as described previously (25).

Polyacrylamide gel electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out by using the buffers of Laemmli (12) and included 4.5% stacking and 12.5 or 7.5% running gels. Gels were electroblotted onto nitrocellulose, probed with the appropriate antibodies, and visualized by using donkey anti-rabbit horseradish peroxidase and enhanced chemiluminescence detection reagents (Amersham, Arlington Heights, Ill.). Antipeptide antibodies against the N-terminal 15 amino acids of MgtB and the C-terminal 16 residues of MgtC were prepared by Quality Controlled Biochemicals (Hopkinton, Mass.) and raised in rabbits. The internal control was an unknown protein that cross-reacted similarly with both the preimmune serum and serum containing the anti-MgtC antibodies. The gels shown have been scanned in Adobe Photoshop. Contrast has not been adjusted.

Disk diffusion assays for cation sensitivity. Strains were grown overnight at 37°C in Luria-Bertani (LB) broth supplemented with 10 mM Mg^{2+}. Cells were washed twice with an equal amount of LB. The cell pellet was resuspended in 5 ml of LB, and 100 μl was spread onto an LB plate. A 6-mm-diameter Whatman filter paper disk was placed in the center of the plate and loaded with 15 μl of 1 M cation solution ($CoCl_2$, $NiCl_2$, $ZnCl_2$, $MnCl_2$, $CaCl_2$, and $SrCl_2$). The plates were incubated at 37°C for 24 or 48 h. Sensitivity was measured as the area of the clear growth inhibition ring minus the 18-mm^2 area of the disk.

Atomic absorption. A 1.0-ml volume of cells was layered over 0.3 ml of a 2:1 mixture of dibutyl and dioctyl phthalate and spun in a microcentrifuge for 30 s. The supernatant was carefully aspirated, the sides of the tube were swabbed with a cotton-tipped applicator, and 0.1 ml of 1.0 N HNO_3 was added. The sealed tubes were batch sonicated for 30 s, and the divalent cation content was measured by atomic absorption as described previously (20).

RESULTS

Requirement of *mgtCB* for growth. As controls to confirm previous results (3) that *mgtC* is necessary for optimal growth at low extracellular Mg^{2+} concentrations, cells lacking a functional *mgtCB* operon (MM196 [*mgtC mgtB*]) or lacking *mgtB* (MM197 [*mgtC*$^+$ *mgtB*]) were grown in N minimal medium with high or low Mg^{2+}. Strains grown in 10 mM Mg^{2+} exhibited a similar OD_{600} in the presence or absence of *mgtC* (Fig. 1A). When cells were grown in 10 μM Mg^{2+} (Fig. 1B), the *mgtC mgtB* mutant reached an OD_{600} considerably lower than that of the wild-type strain. The *mgtC*$^+$ *mgtB* mutant grew to a higher OD_{600} than the *mgtC mgtB* mutant but less than that of the wild type. These results are similar to those observed by Blanc-Potard and Groisman (3), suggesting that both genes of the *mgtCB* locus are necessary for optimal growth at low Mg^{2+} concentrations.

To determine if *S. typhimurium* could grow in the presence of *mgtC* with or without a functional *mgtB* allele but in the absence of the other known Mg^{2+} transporter genes of *S. typhimurium*, we used strain MM281 (*corA mgtA mgtC mgtB*) transformed with plasmid pMBM19, which contains a functional *mgtC* allele. The resulting strain MM1665 was grown in N minimal medium supplemented with various Mg^{2+} concentrations. At 10 mM Mg^{2+}, this *mgtC*$^+$ *mgtB* strain grew to a higher OD_{600} than an isogenic strain (MM1490) carrying the intact *mgtCB* operon (*mgtC*$^+$ *mgtB*$^+$ [Fig. 2A]). At a lower Mg^{2+} concentration of 70 μM, the *mgtC*$^+$ *mgtB* strain showed no growth for 24 h but then grew to a final OD_{600} of 0.45 after

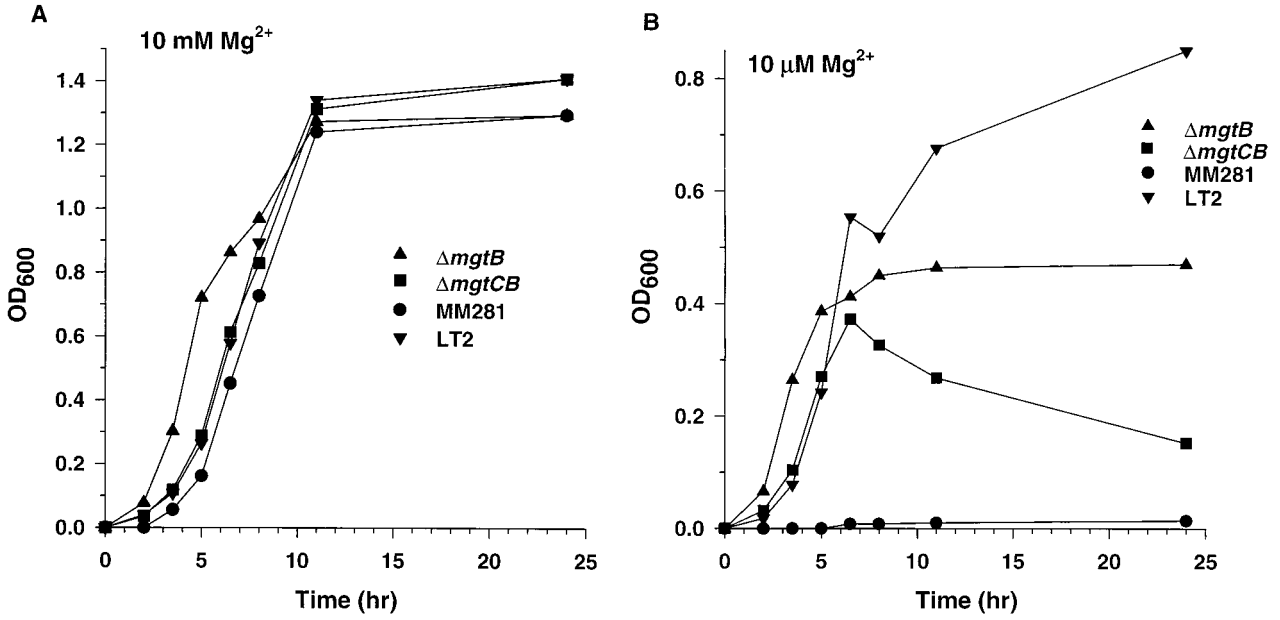

FIG. 1. Growth curves of strains possessing or lacking a functional *mgtC*. Strains MM1442 (wild-type LT2) (▼), MM281 (*corA mgtA mgtC mgtB*) (●), MM196 (*mgtC mgtB*) (■), and MM197 (*mgtC*$^+$ *mgtB*) (▲) were grown overnight in N minimal medium supplemented with 10 mM or 10 μM Mg^{2+}. Cultures were washed three times with N minimal medium without added Mg^{2+} and resuspended in a volume to give the same initial optical density (OD_{600}). The samples were diluted 1:200 in N medium containing various Mg^{2+} concentrations and incubated at 37°C. Aliquots were removed at the indicated times, and the OD_{600} was measured. Data similar to that at 10 μM Mg^{2+} were obtained at Mg^{2+} concentrations below 0.5 mM.

48 h. MM1490 carrying the intact operon exhibited a shorter though still increased lag phase and reached stationary phase at a higher OD_{600} by 24 h (Fig. 2B). Similar time courses were obtained at lower Mg^{2+} concentrations, although the maximal OD_{600} was lower. This slow growth of a strain carrying only an *mgtC*$^+$ allele is also evident on rich media (Fig. 3). Growth of the *mgtC*$^+$ *mgtB* strain (MM1665) after 24 h at 37°C is barely detectable, while both the *mgtC*$^+$ *mgtB*$^+$ (MM1490) and the *mgtC mgtB*$^+$ (MM1733) strains exhibit substantial growth. MM1665 (*mgtC*$^+$ *mgtB*) strain shows modest growth when incubation is continued for an additional 24 h (data not shown).

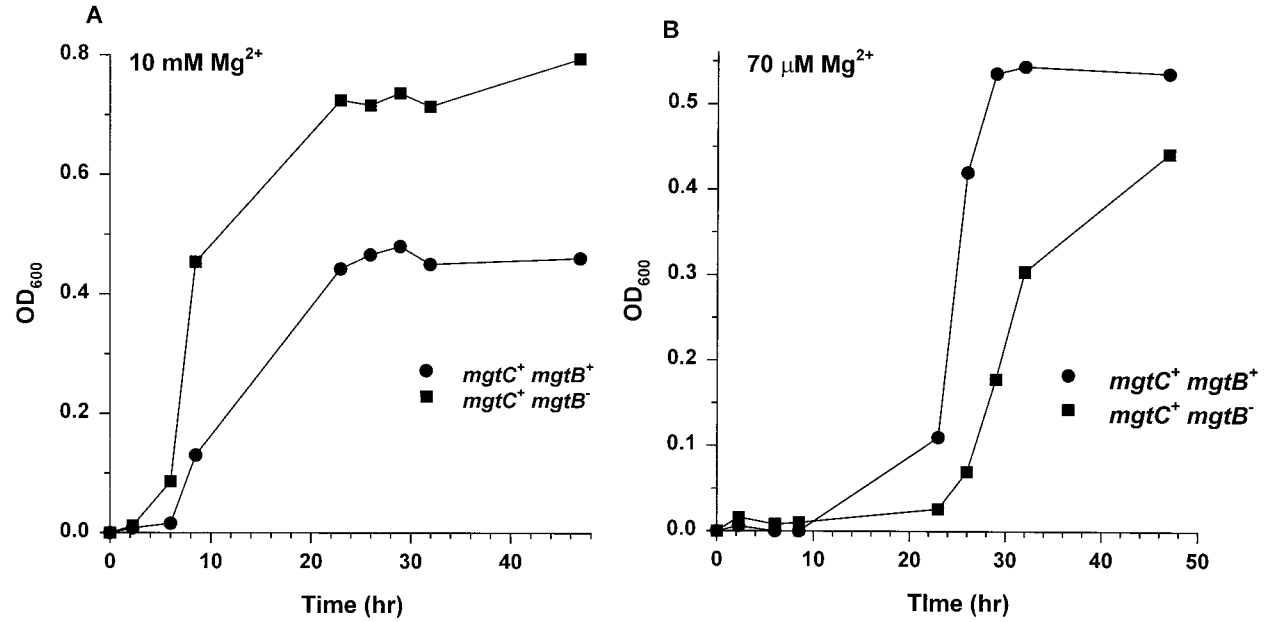

FIG. 2. Growth analysis of strains grown in the presence of absence of *mgtB*. Cultures of MM1490 (*mgtC*$^+$ *mgtB*$^+$) (●) and MM1665 (*mgtC*$^+$ *mgtB*) (■) were grown overnight in N minimal medium supplemented with 10 mM or 70 μM Mg^{2+}. Cultures were processed as described in the legend to Fig. 1 and grown at various Mg^{2+} concentrations at 37°C. Aliquots were removed at the indicated times, and the OD_{600} was measured. Growth similar to that seen at 70 μM Mg^{2+} was seen at other Mg^{2+} concentrations below 500 μM.

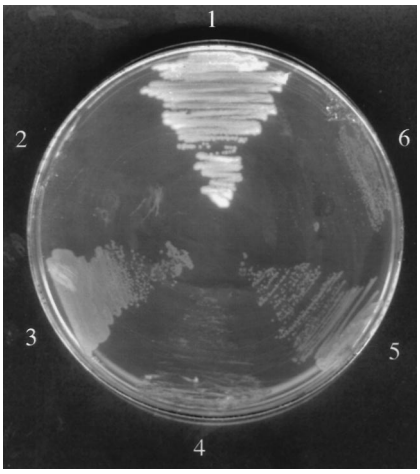

FIG. 3. Growth phenotype of the strain carrying only *mgtC*. LB plates were streaked with the following strains and incubated at 37°C for 24 h. Strains: 1, LT2 (wild type); 2, MM281 (*corA mgtA mgtC mgtB*); 3, MM1490 (*mgtC*$^+$ *mgtB*$^+$); 4, MM1665 (*mgtC*$^+$ *mgtB*); 5, MM1733 (*mgtC mgtB*$^+$); 6, MM1542 (*mgtC*$^+$ *mgtB*[D379A]) The plate was scanned and contrast was adjusted by using Adobe Photoshop 4.0.

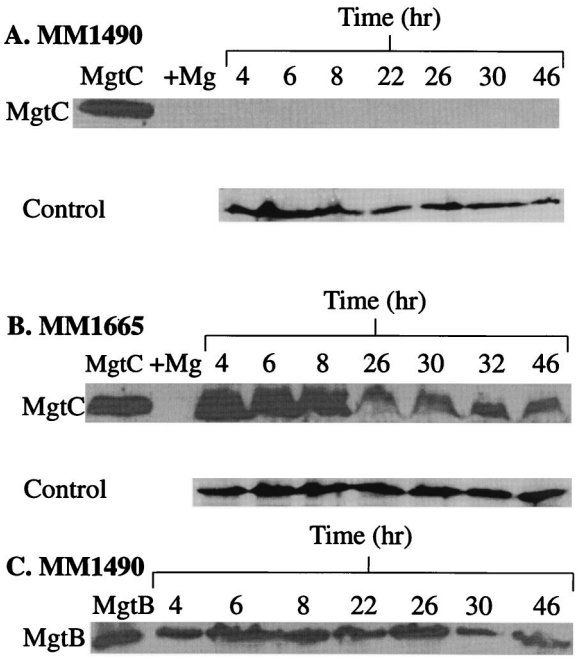

FIG. 4. Effect of mgtB on the detection of MgtC protein in whole cells. Strains MM1490 (A and C) and MM1665 (B) were grown overnight in 10 mM Mg^{2+}, washed three times in N minimal medium without added Mg^{2+}, resuspended in the same medium in the absence of Mg^{2+}, and incubated at 37°C. Cell aliquots were removed at the indicated time points and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 12.5 or 7.5% polyacrylamide gels followed by transfer to nitrocellulose. Proteins were visualized with anti-MgtC (A and B) or anti-MgtB (C) antibodies as described in Materials and Methods. The internal control was a band that reacted with both the preimmune serum and the anti-MgtC antibody.

To confirm that growth after such a long lag phase was not due to a revertant, pMBM19 DNA was isolated from cells grown in 10 μM Mg^{2+} for 48 h and used to retransform MM281. The growth curve of this strain was similar to that of the original MM1665 strain (data not shown). In addition to this experiment, a single colony of MM1665 was taken from an LB plate supplemented with 100 mM Mg^{2+}, grown for 48 h on an LB plate lacking additional Mg^{2+}, and tested for growth in liquid culture at various Mg^{2+} concentrations. These results were also similar to the growth curves shown for MM1665 in Fig. 2B (data not shown).

Absence of MgtC in strains containing MgtB. Western blot analysis was performed on *mgtC*$^+$ *mgtB*$^+$ strains versus *mgtC*$^+$ *mgtB* strains to determine if MgtC protein was being produced. MM1665 (*mgtC*$^+$ *mgtB*) expressed significant quantities of MgtC by 2 h after initiation of Mg^{2+} starvation, and MgtC was still detectable after 48 h (Fig. 4B). Surprisingly, no significant quantities of MgtC were detected in the MM1490 strain carrying the intact *mgtCB* operon (Fig. 4A) or in the wild-type LT2 strain (data not shown) after Mg^{2+} starvation. MgtB, which is encoded by the second gene of the operon, is expressed after Mg^{2+} starvation in MM1490 (Fig. 4C) and LT2 but not MM1665 (data not shown).

Since MgtC is not expressed in detectable quantities in the presence of a wild-type allele of *mgtB*, we examined whether an MgtB protein capable mediating of Mg^{2+} transport was necessary for this effect. MgtB is a P-type ATPase. This family of transporters has a characteristic aspartate (D379 in MgtB) at its active site which becomes phosphorylated during cation transport. If the aspartate is changed to another amino acid, phosphorylation cannot occur and the protein is nonfunctional. An D379A MgtB protein carried in MM1542 cannot transport Mg^{2+} and does not complement the Mg^{2+} transport-deficient strain MM281 (11a). This strain was starved for Mg^{2+} as described above. At 2 h after initiation of Mg^{2+} starvation, some MgtC could be detected in the cells (Fig. 5A). MgtC was, however, almost absent by 16.5 h after Mg^{2+} starvation. This contrasts with the results in the MM1490 (*mgtC*$^+$ *mgtB*$^+$) strain, where there was no detectable MgtC in whole cells at 2 h (data not shown) or 4 h (Fig. 4A) after Mg^{2+} starvation. Although nonfunctional, the D379A MgtB protein was readily expressed 2 h after initiation of Mg^{2+} starvation of strain MM1542 (Fig. 5B) and was still present in cells after 49 h of Mg^{2+} starvation. Consistent with these data, MM1542 is unable to grow significantly on plates in rich medium (Fig. 3). These results suggest that MgtC protein is not produced or is not stable in the presence of MgtB protein, regardless of whether MgtB can function as a Mg^{2+} transporter.

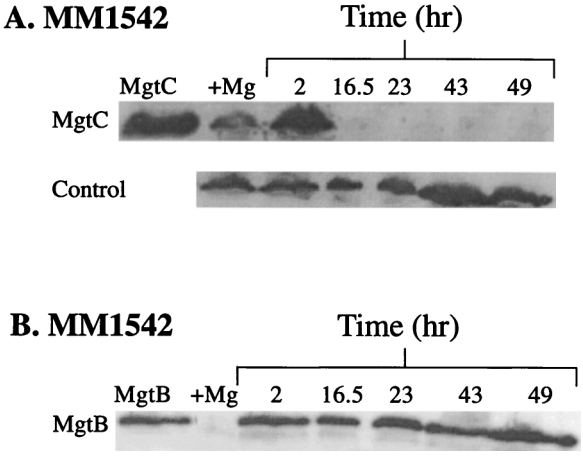

FIG. 5. MgtC detection in an D379A MgtB mutant. Strain MM1542 was grown and samples were processed as described in the legend to Fig. 4.

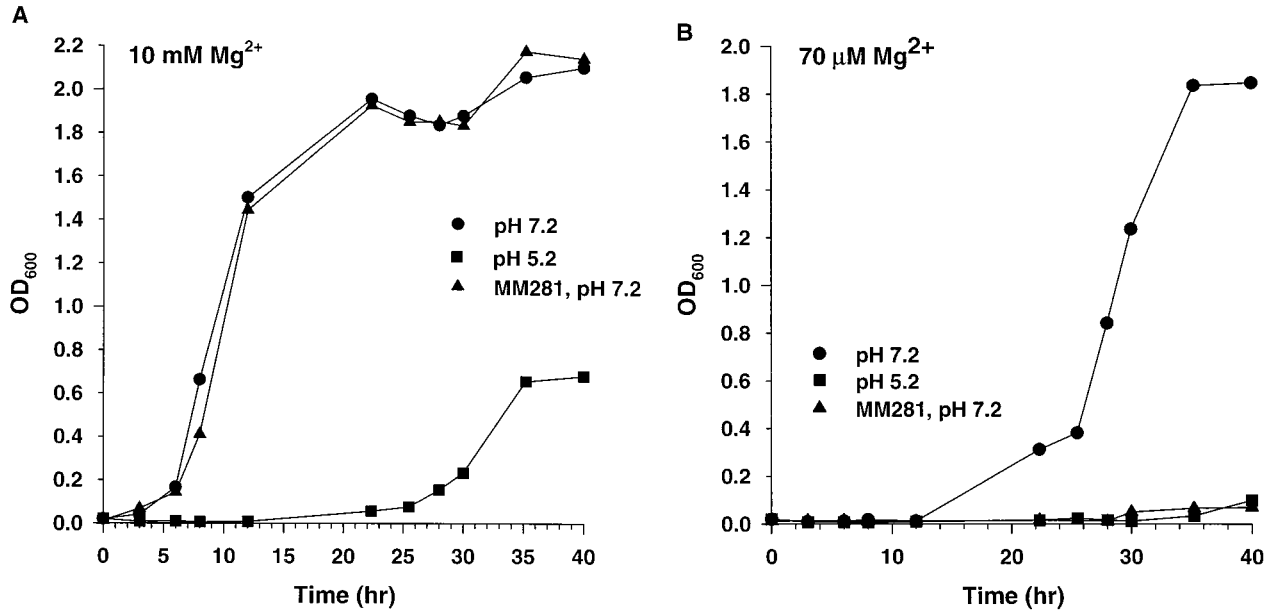

FIG. 6. Effects of pH 5.2 and low Mg^{2+} concentration on growth in the presence of *mgtC*. MM1665 (●, ■) and MM281 (▲) were grown overnight in N minimal medium supplemented with 10 mM or 70 μM Mg^{2+}. Cultures were washed with N minimal medium (pH 7.4 or pH 5.2) three times and resuspended in a volume to give the same initial OD_{600}. The samples were diluted 1:200 in N minimal medium at pH 7.2 (●) or pH 5.2 (■) containing various Mg^{2+} concentrations and incubated at 37°C. Aliquots were removed at the various time points, and the OD_{600} was measured. Growth of MM281 at pH 7.4 was used as a negative control. Data similar to that shown for 70 μM Mg^{2+} were obtained at Mg^{2+} concentrations below 500 μM.

Growth of an *mgtC*$^+$ strain at low pH. When *S. typhimurium* bacteria invade epithelial or macrophage cells, they encounter an environment with a low pH in addition to one low in Mg^{2+} (7). To investigate whether *mgtC* might be involved in *S. typhimurium* survival at low pH, MM1665 (*mgtC*$^+$ *mgtB*) was grown at pH 7.5 or 5.2 in N minimal medium supplemented with various concentrations of Mg^{2+}. In the presence of 10 mM Mg^{2+}, cells grown at pH 7.4 grew to an OD_{600} similar to strain MM281 (Fig. 6A). At pH 5.2 with 10 mM Mg^{2+}, the *mgtC*$^+$ *mgtB* strain exhibited a markedly increased lag phase and grew to a lower OD_{600}. At lower Mg^{2+} concentrations, an *mgtC*$^+$ *mgtB* strain could not grow to any significant extent at pH 5.2 (Fig. 6B).

To determine whether the lack of growth of MM1665 at low pH was due to the lack of MgtC, we examined its expression. At pH 5.2 in the absence of Mg^{2+}, MgtC was readily detectable in the *mgtC*$^+$ *mgtB* strain (Fig. 7A) and was produced at levels similar if not greater than those in cells starved for Mg^{2+} at pH 7.2 (Fig. 4A). In the *mgtC*$^+$ *mgtB*$^+$ strain (MM1490), MgtC was detected after 4 h of Mg^{2+} starvation but was not detected at significant levels at 8 h or at later times (Fig. 7B). This result is similar to the result with the D379A MgtB mutant (Fig. 5A). At pH 5.2, a small amount of MgtB protein was detected in MM1490 at 4 h after initiation of Mg^{2+} starvation, and its expression increased greatly with increasing time of incubation. The opposite accumulation of MgtC versus MgtB protein contrasts with the transcriptional data for this operon after exposure to acid. At 2 to 4 h after acute exposure to pH 5.2 and low extracellular Mg^{2+} concentrations, transcription of *mgtCB* is severely diminished, but adaptation to acid conditions overnight restores full transcriptional response (28). This is consistent with the initially low expression of MgtB protein, whereas expression of MgtC is again anomalous.

The studies described above were performed in an *S. typhimurium* LT2 background which is known to carry a mutation in *rpoS*, which encodes a stationary-phase sigma factor (31). We have previously shown that the presence or absence of a functional *rpoS* allele has no effect on Mg^{2+} or pH regulation of *mgtA* or *mgtCB* transcription (28). To determine if *rpoS* had any effect on levels of MgtC protein, we transferred pMBM19 (*mgtC*$^+$) into MM1266 (*mgtC mgtB*) which is derived from an *S. typhimurium* 14028s background. This strain also carries wild-type *corA* and *mgtA* alleles and thus does not require Mg^{2+} supplementation for growth. When this strain (MM1737) was grown in N minimal medium without supplemental Mg^{2+}, there was a substantial increase in MgtC protein over time (Fig. 7D). The amount of MgtC present was comparable to that seen in the LT2 background (Fig. 7A). These results suggest that the presence of MgtC is not dependent on the presence or absence of functional alleles of *corA*, *mgtA*, or *rpoS*. In contrast, MgtC is absent in the presence of MgtB protein, whether or not MgtB is capable of Mg^{2+} influx.

Transport of Ni^{2+} and Co^{2+}. MgtC allows growth of *S. typhimurium* in the absence of added Mg^{2+}. This suggests that it may be involved in Mg^{2+} uptake. Blanc-Potard and Groisman (3) postulated MgtC to be a Mg^{2+} transporter based on the ability of a very high (25 mM) concentration of Mg^{2+} to increase intramacrophage survival of a strain of *S. typhimurium* lacking *mgtC*. We have previously demonstrated that strain MM281, which lacks functional alleles of *mgtC* and the three characterized Mg^{2+} transporters, *corA*, *mgtA*, and *mgtB*, requires a high extracellular Mg^{2+} concentration for growth and cannot take up $^{28}Mg^{2+}$ (25). Unfortunately, this isotope is no longer available; however, *corA*, *mgtA*, and *mgtB* all mediate uptake of $^{63}Ni^{2+}$ as an alternative substrate (25). In addition, *corA* (11, 25) and the *mgtE* Mg^{2+} transporter found in some bacterial species (26, 30) mediate $^{57}Co^{2+}$ uptake. We therefore measured uptake of both of these radioisotopes in a strain carrying only a functional allele of *mgtC*. To induce *mgtC* expression, cells were starved for Mg^{2+} for 6.5, 23, or 47 h. The cells contained significant increases in MgtC content indicated by Western blot analysis at each of these time points (data not

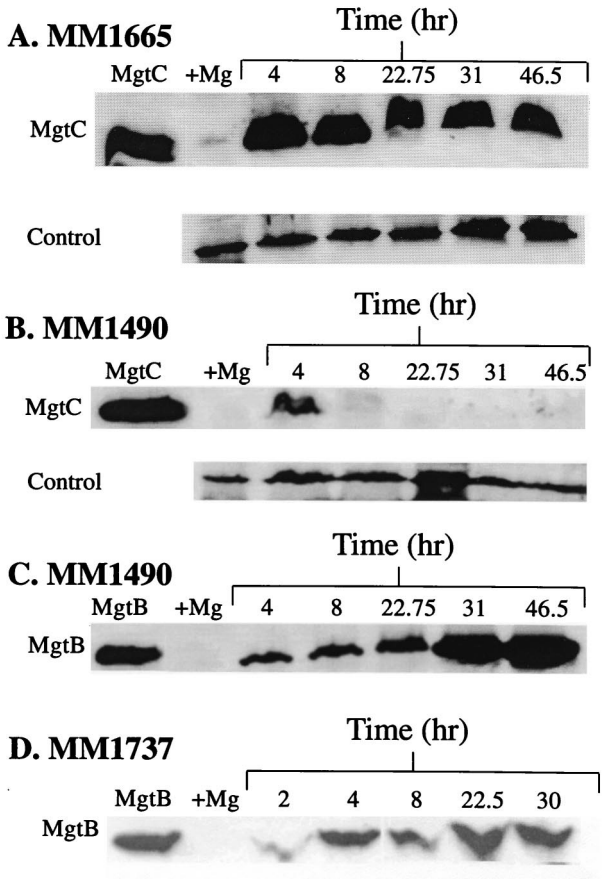

FIG. 7. Effect of pH on the detection of MgtC in whole cells. Strains MM1665 (A), MM1490 (B and C), and MM1737 (D) were grown in N minimal medium in the absence of Mg^{2+} at pH 5.2 (A to C) or pH 7.2 (D). Cell aliquots were removed at the indicated time points for electrophoresis and visualization of MgtC and MgtB proteins was performed as described in the legend to Fig. 4.

shown) similar to that shown in Fig. 4. Uptake was measured at multiple time points, since although MgtC protein is expressed by 2 h after Mg^{2+} starvation, the cultures exhibit a 24-h lag phase before achieving significant growth. Nonetheless, despite the presence of substantial amounts of MgtC protein, no $^{63}Ni^{2+}$ uptake was detectable at any time point (data not shown). With $^{57}Co^{2+}$, an apparent uptake of about 1% that of the wild-type strain could be measured, but this uptake could not be completely inhibited by 100 mM Mg^{2+} (data not shown), suggesting that it was due to nonspecific Co^{2+} binding to the cells.

We also measured Mg^{2+} content of cells with a functional *mgtC* allele (Table 2). The presence of a functional MgtB Mg^{2+} transporter in strain MM1490 increased total cellular Mg^{2+} content significantly compared to MM281. However, strain MM1665 (*mgtC*$^+$ *mgtB*) showed no significant change in Mg^{2+} content when grown in the presence of Mg^{2+} compared to MM281. When grown in the absence of Mg^{2+}, MM1665 showed a slight decrease in Mg^{2+} content, again incompatible with a Mg^{2+} transport function for MgtC. Finally, cation sensitivity disk diffusion studies for Co^{2+}, Ni^{2+}, Zn^{2+}, Mn^{2+}, Ca^{2+}, and Sr^{2+} showed significant sensitivity in a strain carrying the wild-type *mgtCB* operon on a plasmid. In contrast, strain MM1665 (*mgtC*$^+$ *mgtB*) exhibited significant sensitivity to Ca^{2+} but not to other cations. This sensitivity is probably due to overexpression of MgtC from the plasmid, since strain MM1648, which carries a chromosomal wild-type *mgtC* allele but an inactivated *mgtB* gene, exhibits no apparent sensitivity to Ca^{2+} (data not shown).

DISCUSSION

The *mgtCB* operon forms part of an *S. typhimurium* pathogenicity island, SPI-3. The *mgtC* gene is essential for virulence in a mouse lethality assay and for long-term survival within a macrophage cell line (3) but is not required for efficient invasion or short-term survival within epithelial or macrophage cultured cell lines (24, 28). Neither MgtB nor MgtA assist in MgtC's role in survival within macrophages. The function of the MgtC protein is unknown, however. Blanc-Potard and Groisman (3) have suggested that MgtC is a fourth Mg^{2+} transporter of *S. typhimurium* required for acquisition of Mg^{2+} within the macrophage. This hypothesis is based largely on the ability of an *S. typhimurium mgtC* mutant strain to survive within a macrophage cell line if 25 mM Mg^{2+} is added to the culture medium. Presumably, the high extracellular Mg^{2+} concentration allows the macrophage cell line to increase intracellular Mg^{2+} and thus supply additional Mg^{2+} for intravacuolar growth of *S. typhimurium*. This scenario seems highly unlikely based on the known properties of Mg^{2+} homeostasis in mammalian cells. While Mg^{2+} exchanges readily in some tissues and cell types, including liver, heart, and adipocytes (4, 5, 9, 17, 19), Mg^{2+} uptake and exchange in most cells, including lymphocytes and muscle cells, is severely restricted. In lymphocytes, Mg^{2+} is compartmented and cannot be fully exchanged with the extracellular medium even after 48 h of incubation (9). Moreover, exposure of intravacuolar *S. typhimurium* to an increased free Mg^{2+} concentration would presumably require that cytosolic free Mg^{2+} increase markedly upon addition of extracellular Mg^{2+}. Cytosolic free Mg^{2+} is tightly controlled, however, and simple addition of even high concentrations of extracellular Mg^{2+} does not increase cytosolic free Mg^{2+} in any cell type examined. Thus, it seems highly unlikely that simple addition of extracellular Mg^{2+} would be able to supply significant additional Mg^{2+} to a bacterium within a eukaryotic cell vacuole.

This interpretation is consistent with the inability of a strain expressing only MgtC to accumulate either $^{63}Ni^{2+}$ or $^{57}Co^{2+}$. Since one or both of these cations is transported by all known Mg^{2+} transport proteins (25, 26), it is reasonable to infer that MgtC is not a Mg^{2+} transport protein. Nonetheless, since $^{28}Mg^{2+}$ is no longer available and thus Mg^{2+} uptake cannot be tested directly, a definitive answer to this question must await purification and reconstitution of any transport activity that MgtC might be capable of.

The conclusion that MgtC is not a Mg^{2+} transporter is further consistent with Mg^{2+} effects on the growth of an *mgtC*$^+$ strain carrying mutations in the known Mg^{2+} transport-

TABLE 2. Cell Mg^{2+} content[a]

Strain	Grown in 10 mM Mg^{2+}[b]	Mgt protein expressed	Mg^{2+} content ± SD (nmol/OD_{600})
MM281	+	None	1,397 ± 117
MM1490	+	MgtB	1,916 ± 73
MM1665	+	None	1,253 ± 70
MM1665[c]	−	MgtC	904 ± 49

[a] Mg^{2+} content was measured by atomic absorption as described in Materials and Methods in four individual cell samples. A second experiment gave comparable results.
[b] +, grown in the presence of 10 mM Mg^{2+}; −, grown in the absence of Mg^{2+}.
[c] Cells were starved for Mg^{2+} for 41 h.

ers (*corA mgtA mgtB*). Substantial amounts of MgtC protein are synthesized in such a strain very early after initiation of Mg^{2+} starvation. Nonetheless, no growth occurs for at least 24 h in N minimal medium (Fig. 2B) or rich medium (Fig. 3). If MgtC were a Mg^{2+} transporter, growth should begin relatively early after Mg^{2+} starvation. Nonetheless, despite data indicating that a strain carrying only *mgtC* is impaired in growth, given sufficient time, such strains are able to grow at very low Mg^{2+} concentrations. Although this suggests that the cells are able, eventually, to obtain sufficient Mg^{2+} for growth, Mg^{2+} content by atomic absorption analysis is actually slightly decreased in a strain producing only MgtC, again inconsistent with a Mg^{2+} transport function for this protein. Rather, the ability to grow presumably reflects some unknown adaptation to Mg^{2+} deprivation.

Although the cells do not begin to grow until approximately 24 h after Mg^{2+} starvation, MgtC is synthesized very early after initiation of Mg^{2+} starvation, consistent with regulation studies of the *mgtCB* locus (24, 27–29). Moreover, the amount of MgtC protein did not correlate with growth; it generally remained relatively constant for 48 h after Mg^{2+} starvation was initiated. In sharp contrast, MgtC is virtually undetectable in the presence of a functional MgtB Mg^{2+} transport protein. MgtB could directly interact with MgtC, making it unstable. This would imply some type of protein-protein interaction, perhaps even that MgtC could function as a subunit of MgtB. However, a direct interaction seems unlikely. The *mgtB* and *mgtC* genes are not always associated and can occur together or separately within enterobacteria (3). Moreover, the lack of a functional *mgtC* allele does not affect MgtB membrane insertion or transport properties (29). Nonetheless, the converse conclusion cannot be ruled out. MgtB protein could affect the function of MgtC.

A second possibility is that stability of the *mgtC* mRNA transcript could be affected by the presence of an intact *mgtB* open reading frame. There is evidence for differential regulation of protein encoding segments of a polycistronic message. For example, the arsenical resistance operon (*arsRABC*) of resistance plasmid R773 is transcribed as a single polycistronic mRNA (16). The ArsA and ArsC proteins are produced in amounts much greater than that of the ArsB protein, which is encoded by an intervening segment of the mRNA. Northern blot analysis demonstrated that the initial polycistronic mRNA is processed, yielding messages for ArsA and ArsC. The intervening message encoding ArsB appears to be selectively degraded. This internal instability of the polycistronic mRNA could also be applied to the *mgtCB* locus. The *mgtC*-encoded portion of the transcript might be selectively degraded, while the *mgtB* mRNA species would remain stable and be translated. In the presence of an insertionally inactivated *mgtB* gene or absence of an mRNA encoding MgtB, the *mgtC* mRNA might be more stable.

The issue of MgtC expression is made more complicated by the inability of strains dependent on it to grow at acid pH. When *S. typhimurium* bacteria invade epithelial or macrophage cells, they appear to encounter an environment low in nutrients and pH (1, 7) although pH has not been directly measured. The failure of an $mgtC^+$ strain to grow at pH 5.2 (Fig. 6) even though MgtC is produced (Fig. 7) suggests that induction of *mgtC* transcription and expression of *mgtC* are not required for survival at low pH within the eukaryotic cell. This is consistent with our observations that strains lacking a functional *mgtC* have no defect in invasion efficiency or short-term survival in either epithelial-like or macrophage-like cultured cell lines (24). Alternatively, the lack of growth of the "free-living" bacterium at low pH may simply be an indication that such conditions do not fully mimic intravacuolar conditions. Nonetheless, there is evidence that the intravacuolar pH increases with time after bacterial entry into the macrophage (1). Therefore, perhaps the inability of the $mgtC^+$ strain to grow at low pH is not a defect at all but simply reflects that the requirement for a functional *mgtC* occurs late in the invasion process.

MgtC is crucial for *S. typhimurium* virulence. Its regulation by Mg^{2+} via the two-component PhoPQ regulatory system and its chromosomal association with *mgtB*, encoding a Mg^{2+} transporter, might suggest that its function involves Mg^{2+}. Overall, our data do not support this association. It does not appear to function as a Mg^{2+} transporter. At a pH apparently encountered by the bacterium during the pathogenic process, it cannot support growth. Additional studies will need to address the relationship of MgtC to Mg^{2+} and the actual function of MgtC, both in the free-living bacterium and during bacterial encounters with eukaryotic cells.

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